TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 USC 371

International Application No..	PCT/BE00/00081
International Filing Date:	July 11, 2000
Priority Date Claimed:	July 12, 1999
Title of Invention:	NON-HUMAN GENETICALLY MODIFIED MAMMAL LACKING THE ALPHA-FETOPROTEIN
Applicant(s) for DO/EO/US.	GABANT, Philippe ROSCAM-SZPIRER, Josiane

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. (X) This is a **FIRST** submission of items concerning a filing under 35 USC 371.

2. () This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 USC 371

3. (X) This express request to begin national examination procedures (35 USC 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 USC 371(b) and PCT Articles 22 and 39(1).

4. (X) A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. (X) () A copy of the International Application as filed (35 USC 371(c)(2))

   a) () is transmitted herewith (required only if not transmitted by the International Bureau).
   b) (X) has been transmitted by the International Bureau.
   c) () a copy of Form PCT/1B/308 is enclosed.
   d) () is not required, as the application was filed in the United States Receiving Office (RO/US).

6. () A translation of the International Application into English (35 USC 371(c)(2)).

7. (X) A copy of Amended Claims made to PCT Application.

8. () A translation of the amendments to the claims under PCT Article 19 (35 USC 371(c)(3)).

9. () An oath or declaration of the inventor(s) (35 USC 371(c)(4)).

10. () A translation of the annexes, such as any amendments made under PCT Article 34, to the International Preliminary Examination Report under PCT Article 36 (35 USC 371(c)(5)).

11. () An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. () An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. (X) A FIRST preliminary amendment.

() A SECOND or SUBSEQUENT preliminary amendment.

14. () A substitute specification.

15. () A power of attorney and/or address letter.

16. (X) International Application as published and International Search Report.

17. () The present application qualifies for small entity status under 37 C.F.R. § 1.27.

18. (X) A return prepaid postcard.

19. (X) The following fees are submitted:

				FEES
	BASIC FEE			$890
CLAIMS	**NUMBER FILED**	**NUMBER EXTRA**	**RATE**	
Total Claims	14 - 20 =	0 ×	$ 18	$ 0
Independent Claims	5 - 3 =	2 ×	$ 84	$168
Multiple dependent claims(s) (if applicable)			$280	$ 0
	TOTAL OF ABOVE CALCULATIONS		$1058	
Reduction by 1/2 for filing by small entity (if applicable). Verified Small Entity statement must also be filed. (NOTE 37 CFR 1.9, 1.27, 1.28)			$	
	TOTAL NATIONAL FEE			$ 1058
	TOTAL FEES ENCLOSED			$ 1058

20. (X) The fee for later submission of the signed oath or declaration set forth in 37 CFR 1.492(e) will be paid upon submission of the declaration.

21. (X) A check in the amount of $1058 to cover the above fees is enclosed.

22. () Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40 per property.

23. (X) The Commissioner is hereby authorized to charge only those additional fees which may be required, now or in the future, to avoid abandonment of the application, or credit any overpayment to Deposit Account No. 11-1410.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Daniel Hart

Daniel Hart
Reg. No. 40,637
Customer No. 20,995

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VANM243.1APC1

PATENT #11/13

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

With Statement

Applicant	:	Gabant, et al.	)	Group Art Unit 1632
Appl. No.	:	10/031,021	)	
Filed	:	January 14, 2002	)	
For	:	NON-HUMAN GENETICALLY MODIFIED MAMMAL LACKING THE ALPHA-FETOPROTEIN	)	
Examiner	:	Unknown	)	

SUPPLEMENTAL PRELIMINARY AMENDMENT and SEQUENCE SUBMISSION STATEMENT

United States Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

Dear Sir:

Prior to examination of the above-identified application on the merits, please amend the application as follows:

IN THE SPECIFICATION:

On page 5, immediately following paragraph [0016], please insert the following heading and paragraphs:

Brief Description of the Drawings

[0017] This patent or application file contains at least one drawing executed in color. Copies of this patent or application publication with colored drawings will be provided by the Office upon request and payment of the necessary fees. Figures 3 to 6 are executed in color.

[0018] Figure 1A illustrates the targeted disruption of the afp gene to generate alleles which are deleted for most of the sequence of exon 1, for exon 2 and for exon 3.

Figure 1B illustrates the detection of homologous insertion by Southern blot analysis. Figure 1C illustrates a Southern blot analysis of the progeny of homozygous (-/-) chimeric animals.

[0019] Figure 2A is a Northern blot analysis of total RNA from embryonic liver. Figure 2B is a Western blot analysis of embryonic liver and amniotic fluid protein extracts.

[0020] Figures 3A to 3H illustrate expression of the lacZ gene in embryonic and adult tissue. Figures 3A, 3G and 3H illustrate expression in adult liver and gut cells. Figures 3B to 3F illustrate expression of the lacZ gene in embryonic tissues.

[0021] Figure 4A illustrates the structure of the ovary (arrow) and uterus of an adult *afp*lacZ1/lacZ1(-/-) female. Figure 4B illustrates the ovary from a 12 week old *afp*lacZ1/lacZ1 animal. Figure 4C illustrates the ovary from a 12 week old mutated *afp*lacZ1/lacZ1 female. Figure 4D illustrates the general histological structure of *afp*lacZ1/lacZ ovaries. Figure 4E illustrates the structure of wild type ovaries at 4 months.

[0022] Figure 5A illustrates the nucleotide sequence of the afp gene. Figure 5B illustrates the amino acid sequence of the afp protein.

On page 5, please amend paragraph [0017] as follows:

[0017] A clone containing a 129 genomic fragment of AFP loci was isolated from a lambda library. The library was screened with a probe containing the mouse afp promoter. The genomic insert of about 16 kb was subcloned into pKIL-PCR2 (Gabant et al., 1997). The targeting vector (pAFP K.O-1), consists of two recombination arms. The 5' arms (2.5 kb) were generated by polymerase chain reaction (PCR) using the following primers: N-Mer1: agagcggccgcggaagtgacaaagcagaacc (SEQ ID NO: 1) annealing to the Merl sequence of the afp enhancer 1 (Godboute et al. (1988)) and a primer of the X-exon1: agactcgagggatgagggaagcgggtgtg (SEQ ID NO: 2) complementary to the afp exon1. The PCR fragment generated using Pfu polymerase (Stratagene) was cloned in the pCR-blunt vector (Invitrogen).

On page 8, please amend paragraph [0025] as follows:

[0025] Expression of the lacZ reporter gene expression in embryonic and adult tissues was analyzed. As shown in Figure 3 the β-galactosidase activity was detected in predicted embryonic tissues. In the visceral endoderm only patches of cells were observed to turn the reporter strongly on. In the adult tissues tested specific staining was only detected in cells of the liver and in cells of the gut.

On page 8, please amend paragraph [0026] as follows:

[0026] Intercrosses of heterozygotes (*afp* lacZ1/+) gave rise to viable, apparently normal homozygous mutant mice at a Mendelian ratio in CD1 and C57/B16. On the other hand, a significant divergence was observed in the 129 background (Table 1). To determine whether the targeted allele indeed results in a null mutation, total RNA from the liver of embryos was analyzed by Northern blot hybridization (Figure 2A). A strong signal at 2.2kb corresponding to the *afp* transcript was detected in wild type and heterozygous embryos. No signal was detected in RNA samples extracted from homozygous embryonic liver, showing that the recombination disrupted the *afp* transcript in these animals. A Western blot was also performed on embryonic liver and amniotic fluid protein extracts (Figure 2B) and a strong signal was detected with the wild type extracts while no band corresponding to the AFP was visible in the homozygous extracts demonstrating that these animals do not express AFP.

On pages 11-12, please amend paragraph [0029] as follows:

[0029] In order to identify a possible mutation or deletion in the afp gene, a specific genotyping of afp -/- mice by PCR has been developed. Two primers are used for the first afp#1 anneals in the *afp* promoter region (-116 bp to -137 bp: according to the +1 of the mouse *afp* gene). The second primer afp#2 is complementary to the first exon of the mouse *afp* gene (+141 bp to +160 bp: according to the +1 of the mouse *afp* gene): sequence deleted in the *afp* -/- knock-out mouse described in the text.

Sequence afp#1: cccctgctctgttaattattg (SEQ ID NO: 3)

Sequence afp#2: gaaaatagctcccaagtcac (SEQ ID NO: 4)

On page 12, please amend paragraph [0032] as follows:

[0032] For the knock-out mouse for *afp* described in the text: a couple of primers complementary to *lacZ* (*E. coli* gene) can be used.

lacZ#1: acaacgtcgtgactgggaaaac (SEQ ID NO: 5)

lacZ#2: taatgggataggttacgt (SEQ ID NO: 6)

IN THE SEQUENCE LISTING:

Please incorporate the accompanying paper copy of the Sequence Listing immediately following the "**VERSION WITH MARKINGS TO SHOW CHANGES MADE**", into the present application.

REMARKS

In response to the Notification of Missing Requirements dated March 15, 2002, Applicants have provided a paper copy of the Sequence Listing and the Sequence Listing in computer readable format. Applicants have amended the specification to provide a section titled "Brief Description of the Drawings," to provide descriptions of the Figures (1 to 5) filed with the application on January 14, 2002 and to provide a "SEQ ID NO:" for each sequence listed in the specification on page 12. No new matter has been added.

The Notice of Missing Parts indicates that a signed Declaration is required. However, Applicants submitted a signed Declaration and $65 fee (claiming Small Entity Status) to the USPTO on March 19, 2002, four days after the date of the enclosed Notice of Missing Parts. Applicants provide herewith a copy of the signed Declaration and Transmittal mailed to the USPTO on March 19, 2002 and a copy of the return postcard stamped by the USPTO indicating receipt of these documents. Accordingly, Applicants have satisfied the requirement for a signed Declaration.

I. Sequence Submission Statement

A copy of the Sequence Listing in computer readable format as required by 37 C.F.R. § 1.821(e) is submitted herewith.

Applicant has included the serial number of the present application, the names of the inventors, and the client code corresponding to the present application in the Sequence Listing in accordance with U.S. practice. These amendments do not introduce new matter.

As required by 37 C.F.R. § 1.821(f), I hereby declare that the information recorded on the enclosed disk is identical to the printed Sequence Listing submitted herewith. As each of the sequences provided in the Sequence Listing was present in the specification as filed, no new matter has been added.

II. Drawings

Amending Figure 5

Please amend the drawings as indicated in the accompanying Request for Approval of Drawing Changes and redlined version of Figure 5. Applicants have amended the figure to provide sequence identification numbers for the two sequences listed within the figure. The nucleotide sequence has been identified as SEQ ID NO: 7 and the amino acid sequence has been identified as SEQ ID NO: 8. As each of these sequences were present in the application as filed, no new matter has been added. Applicants respectfully request approval for the changes to Figure 5.

Approval for Submitting Color Photographs

Applicants are submitting herewith color photographs of Figure 3A-H and Figure 4A-E (in triplicate) accompanied by a Petition to Accept Color Photographs as Part of a Utility Patent. Applicants submit that these photographs are the color representation of the black and white figures submitted in the application filed on January 14, 2002 and that no new matter has been introduced. Applicants respectfully request approval for submitting color photographs for formal Figures 3A-H and 4A-E.

III. Conclusion

Applicants have provided herewith a printed version of the Sequence Listing and a copy of the Sequence Listing in computer readable format. The specification has been amended to provide a sequence identification number after each sequence listed in the specification on page 12. Applicants have requested approval for amending

Figure 5 in order to provide a sequence identification number for each sequence in the figure. These amendments mirror the sequences represented in the Sequence Listing submitted herewith. Finally, Applicants have requested approval for submitting color photographs as formal Figure 3A-H and Figure 4A-E.

The changes made to specification by the current amendment, including insertions and **[deletions]**, are shown on an attached sheet entitled **VERSION WITH MARKINGS TO SHOW CHANGES MADE**, which follows the signature page of this amendment. No new matter has been added herewith.

If any issues remain that may be addressed by a phone conversation, the Examiner is invited to contact the undersigned at the phone number listed below.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: July 3, 2002

By: Daniel Hart

Daniel Hart
Registration No. 40,637
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(619) 235-8550

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the specification

On page 5, immediately following paragraph [0016], the following heading and paragraphs have been inserted:

Brief Description of the Drawings

[0017] This patent or application file contains at least one drawing executed in color. Copies of this patent or application publication with colored drawings will be provided by the Office upon request and payment of the necessary fees. Figures 1 to 4 are executed in color.

[0018] Figure 1A illustrates the targeted disruption of the afp gene to generate alleles which are deleted for most of the sequence of exon 1, for exon 2 and for exon 3. Figure 1B illustrates the detection of homologous insertion by Southern blot analysis. Figure 1C illustrates a Southern blot analysis of the progeny of homozygous (-/-) chimeric animals.

[0019] Figure 2A is a Northern blot analysis of total RNA from embryonic liver. Figure 2B is a Western blot analysis of embryonic liver and amniotic fluid protein extracts.

[0020] Figures 3A to 3H illustrate expression of the lacZ gene in embryonic and adult tissue. Figures 3A, 3G and 3H illustrate expression in adult liver and gut cells. Figures 3B to 3F illustrate expression of the lacZ gene in embryonic tissues.

[0021] Figure 4A illustrates the structure of the ovary (arrow) and uterus of an adult *afp*lacZ1/lacZ1(-/-) female. Figure 4B illustrates the ovary from a 12 week old *afp*lacZ1/lacZ1 animal. Figure 4C illustrates the ovary from a 12 week old mutated *afp*lacZ1/lacZ1 female. Figure 4D illustrates the general histological structure of *afp*lacZ1/lacZ ovaries. Figure 4E illustrates the structure of wild type ovaries at 4 months.

[0022] Figure 5A illustrates the nucleotide sequence of the afp gene. Figure 5B illustrates the amino acid sequence of the afp protein.

On page 5, paragraph [0017] has been as follows:

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On pages 11-12, paragraph [0029] has been amended as follows:

[0029] In order to identify a possible mutation or deletion in the afp gene, a specific genotyping of afp -/- mice by PCR has been developed. Two primers are used for the first afp#1 anneals in the *afp* promoter region (-116 bp to -137 bp**[)]**: according to the +1 of the mouse *afp* gene). The second primer afp#2 is complementary to the first exon of the mouse *afp* gene (+141 bp to +160 bp: according to the +1 of the mouse *afp* gene): sequence deleted in the *afp* -/- knock-out mouse described in the text. Sequence afp#1: cccctgctctgttaattattg (SEQ ID NO: 3)
Sequence afp#2: gaaaatagctcccaagtcac (SEQ ID NO: 4)

On page 8, paragraph [0025] has been amended as follows:

[0025] Expression of the lacZ reporter gene expression in embryonic and adult tissues was analyzed. As shown in Figure **[2]**3 the β-galactosidase activity was detected in predicted embryonic tissues. In the visceral endoderm only patches of cells were observed to turn the reporter strongly on. In the adult tissues tested specific staining was only detected in cells of the liver and in cells of the gut.

On page 8, paragraph [0026] has been amended as follows:

[0026] Intercrosses of heterozygotes (*afp* lacZ1/+) gave rise to viable, apparently normal homozygous mutant mice at a Mendelian ratio in CD1 and C57/B16. On the

other hand, a significant divergence was observed in the 129 background (Table 1). To determine whether the targeted allele indeed results in a null mutation, total RNA from the liver of embryos was analyzed by Northern blot hybridization (Figure **[3]**2A). A strong signal at 2.2kb corresponding to the *afp* transcript was detected in wild type and heterozygous embryos. No signal was detected in RNA samples extracted from homozygous embryonic liver, showing that the recombination disrupted the *afp* transcript in these animals. A Western blot was also performed on embryonic liver and amniotic fluid protein extracts (Figure **[3]**2B) and a strong signal was detected with the wild type extracts while no band corresponding to the AFP was visible in the homozygous extracts demonstrating that these animals do not express AFP.

On page 12, paragraph [0032] has been amended as follows:

[0032] For the knock-out mouse for *afp* described in the text: a couple of primers complementary to *lacZ* (*E. coli* gene) can be used.

lacZ#1: acaacgtcgtgactgggaaaac (SEQ ID NO: 5)

lacZ#2: taatgggataggttacgt (SEQ ID NO: 6)

VANM243.1APC1 PATENT

#13/12

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Gabant, et al.	) Group Art Unit Unknown
Appl. No.	:	Unknown	)
Filed	:	Herewith	)
For	:	NON-HUMAN GENETICALLY MODIFIED MAMMAL LACKING THE ALPHA-FETOPROTEIN	)
Examiner	:	Unknown	)

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Prior to the examination of the application on the merits, please amend the above-referenced application as follows:

IN THE SPECIFICATION

On page 1, immediately following the Title of the Invention, please insert the following heading and paragraph:

Cross-Reference to Related Applications

This is the U.S. National Phase under 35 U.S.C. § 371 of International Application No. PCT/BE00/00081, filed July 11, 2000, which claims priority to U.S. Provisional Application No. 60/143,269, filed July 12, 1999.

IN THE CLAIMS:

On page 16, please replace the centered heading with the following rewritten heading:

WHAT IS CLAIMED IS:

Please cancel Claim 8 without prejudice.

Please amend Claims 1-7 and 9 as follows:

1. **(Amended)** A non-human genetically modified mammal comprising a mutation, a partial deletion or a total deletion in the genetic sequence encoding the wild type alpha-fetoprotein (AFP).

2. **(Amended)** The non-human mammal of claim 1, wherein said non-human mammal is a mouse.

3. **(Amended)** The non-human mammal of claim 1, wherein said non-human mammal is heterozygous for a mutation, a partial deletion or a total deletion in the genetic sequence encoding the wild type alpha-fetoprotein (AFP).

4. **(Amended)** The non-human mammal of claim 1, wherein said non-human mammal is homozygous for a mutation, a partial deletion or a total deletion in the genetic sequence encoding the wild type alpha-fetoprotein (AFP).

5. **(Amended)** The non-human mammal of claim 4, wherein said non-human mammal is a sterile female.

6. **(Amended)** The non-human mammal of claim 4, wherein said non-human mammal does not undergo a menstrual cyclization.

7. **(Amended)** The non-human mammal of claim 4, wherein said non-human mammal does not allow an uteral nidification of an embryo.

9. **(Amended)** A pluripotential embryonic stem cell comprising a partial or a total deletion of a genetic sequence encoding a mammal alpha-fetoprotein (AFP).

Please add the following new claims:

10. **(New)** A method for identifying an agent for use in preventing osteoporosis, increasing fertility, or preventing conception comprising:

obtaining a non-human genetically modified mammal comprising a mutation, a partial deletion or a total deletion in the genetic sequence encoding the wild type mammal alpha-fetoprotein (AFP);

contacting said genetically modified mammal with said agent; and

determining the effects of said agent on osteoporosis, fertility or contraception in said genetically modified mammal.

11. **(New)** A method for identifying a molecule that is able to bind to alpha-fetoprotein or a portion thereof comprising contacting said alpha-fetoprotein or portion thereof with a molecule and measuring binding of said molecule to said alpha-fetoprotein or portion thereof.

12. **(New)** A composition comprising alpha-fetoprotein or a portion thereof fixed to a solid surface.

13. **(New)** The embryonic stem cell of claim 9, wherein said stem cell is a mouse cell.

IN THE ABSTRACT:

Please insert the abstract on the accompanying separate sheet, immediately following the "**VERSION WITH MARKINGS TO SHOW CHANGES MADE.**"

Conclusion

The specification and claims have been amended to conform with the rules of practice before the U.S. Patent and Trademark Office. The specification has been amended to recite the International Application and priority application. Claim 8 has been cancelled without prejudice. Claims 1-7 and 9 have been amended and new Claims 10-13 have been added. An abstract has been added.

The changes made to specification and claims by the current amendment, including insertions and **[deletions]**, are shown on an attached sheet entitled **VERSION WITH MARKINGS TO SHOW CHANGES MADE**, which follows the signature page of this amendment. No new matter has been added herewith.

Appl. No.	:	**Unknown**
Filed	:	**Herewith**

In view of the foregoing, Applicants respectfully submit that the present application is fully in condition for allowance. Should there be any questions concerning this application, the Examiner is invited to contact the undersigned attorney at the telephone number appearing below.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: Jun 14, 2002

By: Daniel Hart

Daniel Hart
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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the specification:

The following heading and paragraph has been added on page 1, immediately following the Title of the Invention:

Cross-Reference to Related Applications

This is the U.S. National Phase under 35 U.S.C. § 371 of International Application No. PCT/BE00/00081, filed July 11, 2000, which claims priority to U.S. Provisional Application No. 60/143,269, filed July 12, 1999.

In the Claims:

The centered heading on page 16 has been replaced with the following rewritten heading:

WHAT IS CLAIMED IS:

Claims 1-7 and 9 have been amended as follows:

1. **(Amended)** A non-human genetically modified mammal comprising a mutation, a partial deletion or a total deletion in the genetic sequence encoding the wild type alpha-fetoprotein (AFP).

2. **(Amended)** The non-human mammal **[according to]**of claim 1, **[which is]**wherein said non-human mammal is a mouse.

3. **(Amended)** The non-human mammal **[according to]**of claim 1, **[which comprises a]**wherein said non-human mammal is heterozygous for a mutation, a partial deletion or a total deletion in the genetic sequence encoding the wild type alpha-fetoprotein (AFP).

4. **(Amended)** The non-human mammal **[according to]**of claim 1, **[which comprises an]**wherein said non-human mammal is homozygous for a mutation, a partial deletion or a total deletion in the genetic sequence encoding the wild type alpha-fetoprotein (AFP).

5. **(Amended)** The non-human mammal **[according to]**of claim 4, **[which]**wherein said non-human mammal is a sterile female.

6. **(Amended)** The non-human mammal **[according to]**of claim 4, **[which]**wherein said non-human mammal does not **[present]**undergo a menstrual cyclization.

7. **(Amended)** The non-human mammal **[according to]**of claim 4, **[which]**wherein said non-human mammal does not allow an uteral nidification of an embryo.

9. **(Amended)** A pluripotential embryonic stem cell**[, preferably a mouse cell,]** comprising a partial or a total deletion of a genetic sequence encoding a mammal alpha-fetoprotein (AFP).

NON-HUMAN GENETICALLY MODIFIED MAMMAL LACKING THE ALPHA-FETOPROTEIN

Abstract of the Disclosure

The present invention is related to a non-human genetically modified mammal comprising a mutation, a partial or total deletion of the genetic sequence encoding the wild type mammal alpha-fetoprotein.

NON-HUMAN GENETICALLY MODIFIED MAMMAL LACKING THE ALPHA-FETOPROTEIN

Field of the invention

[0001] The present invention is related to a non-human genetically modified mammal, preferably a knock-out mouse, comprising a partial or total deletion of a genetic sequence encoding the alpha-fetoprotein (AFP) and used as a model for the study of fertilization or contraceptive methods and drugs.

[0002] The present invention is related to a non-human mammalian pluripotential embryonic stem cell comprising a partial or total deletion of a genetic sequence encoding a mammal alpha-fetoprotein (AFP).

[0003] The present invention is also related to study, testing and/or screening method and device of known or unknown compounds able to bind the mammal alpha-fetoprotein (AFP) and that may be used as agonist or antagonist of oestrogens to the mammal alpha-fetoprotein.

Background of the invention

[0004] Alpha-fetoprotein (AFP) is a glycoprotein present in the serum and a classical oncofetal marker. This protein is expressed at high levels during fetal life in the liver and the visceral endoderm of the yolk sac, and at lower levels in the developing gastrointestinal tract (Andrews et al., 1982; Tilghman and Belayew 1982), in the

adult serum only trace amount are detected (Tilghman and Belayew 1982). The protein expressed by the embryos is secreted and present in the maternal blood circulation during gestation, the level of AFP concentration in the maternal serum is use to detect fetuses with spina bifida or Down's syndrome. The reason for this altered AFP level associated with those pathologies are not understood, but they have been used extensively in prenatal screening. The synthesis of AFP decreases dramatically after birth and only trace amounts are detected in adult liver. However expression of *afp* is associated with hepatocarcinomas and liver regeneration induced by partial hepatectomy or acute tetrachloride (CCl4) intoxication. The control of *afp* gene expression has thus attracted much attention and it has been shown that *afp* expression is regulated by transcriptional mechanisms involving a large promoter and three distant enhancers (review of Chen et al. (1997)). Because AFP is synthesized during the G1 and S phases, it has been hypothesized that intracellular AFP affects cell growth (Leffert and Sell, 1974; Sell et al., 1975; Tsukada and Hirai, 1975; Belanger et al., 1978). The observation that AFP is able to bind estrogen led to the suggestion that AFP plays a role in the control of cell metabolism. In addition to binding estrogen, AFP, like albumin to which it is evolutionary related, is able to bind other steroids and endogenous and exogenous substances such as fatty acids, billirubin and various pharmaceutical agents suggesting that AFP may play a general transportation function. For the fetus, in this respect, AFP could serve as a modulator/modifier of various cell growth regulatory pathways during embryonic and fetal development in vertebrates by interacting and/or binding cytoplasmic chaperone proteins that normally escort nuclear receptors or transcription co-factors through the cytoplasm towards

organelle interfaces (Mizejewski, 1995, 1985). AFP has also been proposed to protect the embryo against the maternal immune system, on the basis of the observation that addition of purified AFP into the culture of splenic or lymphnode mononuclear cells exerts a suppressive effect on antibody synthesis.

[0005] The different hypotheses proposed for AFP function(s) can be focused on the fetal life (stage at which the gene is strongly transcripted) since the protein is described as a fetoprotein.

[0006] At the present time, no document of the state of the art has suggested that the alpha-fetoprotein may play an essential role for female reproduction and fertility.

Aims of the invention

[0007] The present invention aims to provide new models (animal models) as well as new methods and devices for the study, the testing and/or the screening of fertilization or contraceptive methods, compounds and compositions intended for adult mammals (including humans) and/or for the study, the testing and/or the screening of new methods, compounds or compositions intended for the treatment and/or the prevention of osteoporosis.

Summary of the invention

[0008] The present invention is related to a non-human genetically modified mammal (preferably a knock-out mouse) comprising a mutation, a partial or total deletion in a genetic sequence encoding a mammal alpha-fetoprotein (AFP) described in GenBank (v00743).

[0009] Advantageously, said mammal comprises an heterozygous or homozygous mutation, partial or total deletion in the genetic sequence encoding a mammal AFP.

[0010] According to another embodiment of the present invention, said mammal is a sterile female comprising said homozygous mutation, partial or total deletion.

[0011] The present invention is also related to specific sequences such as primers that are used to identify if a mammal comprises said mutation, partial or total deletion heterozygously or homozygously.

[0012] Another aspect of the present invention is related to a non-human mammal pluripotential embryonic stem cell, preferably a mouse pluripotential embryonic stem cell comprising a partial or total deletion of a genetic sequence encoding a mammal AFP. Said stem cell can be advantageously used to obtain the non-human genetically modified mammal according to the invention by methods well known by the person skilled in the art described hereafter.

[0013] A further aspect of the present invention is related to the use of the non-human mammal according to the invention for the study, the testing and/or the screening of known or unknown anti-osteoporosis fertilization and/or contraceptive methods, compounds or compositions.

[0014] A last aspect of the present invention is related to study, testing and/or screening methods and devices comprising the AFP or a portion of said AFP, preferably the AFP domain III comprising about 200 amino acids (as described by Festin (1999)), being fixed upon a solid support and used as a substrate for known or unknown compounds or compositions in a competitive test or method. Said device comprises also a medium comprising (possibly labeled) oestrogens. The device according to the invention can be a chromatographic column upon which the AFP or the portion thereof is fixed or a study, testing and/or screening kit comprising disposed separately the various media necessary for said study, testing and/or screening.

[0015] According to a preferred embodiment of the present invention, said device or kit may comprise a cell having integrated an oestrogen-sensitive (proloactine) promoter gene whose activation may result from the fixation of known or unknown compounds or compositions upon the AFP. Said known or unknown compound or composition could be used advantageously as an agonist of an oestrogen.

[0016] The present invention is also related to this unknown agonist or antagonist of oestrogens screened and identified by the method and the device according to the invention. This unknown molecule finds an application in the field of fertilization and/or contraceptive methods and compositions and/or is used also for the treatment and/or the prevention of osteoporosis.

Detailed description of the present invention

Generation of mice carrying a germ-line mutation in the AFP gene

[0017] A clone containing a 129 genomic fragment of AFP loci was isolated from a lambda library. The library was screened with a probe containing the mouse afp promoter. The genomic insert of about 16 kb was subcloned in pKIL-PCR2 (Gabant et al., 1997). The targeting vector (pAFP K.O-1), consist of two recombination arms. The 5' arms (2.5 kb) was generated by polymerase chain reaction (PCR) using the following primers: N-MerI: agagcggccgcggaagtgacaaagcagaacc annealing to the MerI sequence of the afp enhancer 1 (Godboute et al. (1988)) and a primer of the X-exon1: agactcgagggatgagggaagcgggtgtg complementary to the afp exon1. The PCR fragment generated using Pfu polymerase (Stratagene) was cloned in the pCR-blunt vector (Invitrogen).

[0018] The 3' arms were subcloned from the lambda into pBSIIKS+ vector (Stratagene). The 5' recombination arm

was introduced upstream the 3' recombination arm. The IRES lacZ/neo reporter-selective cassette was introduced between these recombination arms. The tk2 negative selective marker was introduced into the SalI site to generate pAFP KO-1. This construction was linearized with NotI and electroporated into E14 ES cells. Correctly targeted clones were identified by Southern blot analysis using an external probe from the 5' region.

ES cell injections and animal genotyping

[0019] Recombinant ES cells carrying the targeted allele were injected in C57BL/6J blastocysts. Animals were genotyped by extraction of DNA from tails.

RNA isolation, Northern blot analysis

[0020] Total RNA was isolated using Trizol (Gibco BRL) extraction according to the manufacturer instructions. For the Northern analysis 20µg of total RNA were electrophoresed and transferred to nylon membranes as described. Filters were then hybridized.

Western blot analysis

[0021] Proteins were separated by SDS-PAGE using 7.5% polyacrylamide gels in a Bio-Rad Mini Protean gel chamber and blotted onto Nitrocellulose filters in a Bio-Rad Trans Blot chamber according to the manufacturer's instructions. Proteins were detected using anti-AFP, anti-Albumin; anti Betagalactosidase serum (ICN Biochemicals) the signal was detected with ECL detection system (Amersham).

LacZ reporter gene expression

[0022] To isolate embryonic stages, natural matings were set up and presence of a vaginal plug at noon the following day was taken as 0.5 days of gestation. Staged embryos were stained with X-Gal as wholemounts as described by Forrester et al. (1996). For cryostat sectioning, tissues were embedded in optimal cutting temperature (OTC) compounds (Miles, Inc., Elkart, IN), and sections stained for X-Gal were counterstained with haematoxylin and eosin, and mounted.

Targeted mutagenesis of the afp gene

[0023] The *afp* gene was disrupted by gene targeting in embryonic stem (ES) cells. The *lacZ* reporter was introduced in *afp* gene by homologous recombination and placed under the control of the AFP promoter-enhancer region. The resulting allele is deleted for most of the sequence of exon1, for exon2 and 3 (see figure 1A) and homologous insertion was detected by Southern analysis (see figure 1B). To test the functionality of the reporter one may take advantage of the observation that AFP is expressed in embryoid bodies (Abe et al., 1996). Reporter gene activity is highly turn on in some cells of these bodies (see figure 2A). No expression of the reporter was detected in undifferentiated ES cells grown in the presence of LIF.

[0024] ES cells *afp* $^{lacZ1/+}$ were injected into C57BL/6J blastocysts. Chimeric animals were obtained and mated with outbred CD1 or inbred 129/CGR to test for germ line transmission. Phenotypically normal heterozygous mice *afp* $^{lacZ1/+}$ were generated and detected by Southern blot (see figure 1C).

Reporter expression analysis

[0025] Expression of the lacZ reporter gene expression in embryonic and adult tissues was analyzed. As shown in Figure 2 the β-galactosidase activity was detected in predicted embryonic tissues. In the visceral endoderm only patches of cells were observed to turn the reporter strongly on. In the adult tissues tested specific staining was only detected in cells of the liver and in cells of the gut.

Animals without AFP are viable

[0026] Intercrosses of heterozygotes (*afp* lacZ1/+) gave rise to viable, apparently normal homozygous mutant mice at a Mendelian ratio in CD1 and C57/B16. On the other hand, a significant divergence was observed in the 129 background (Table 1). To determine whether the targeted allele indeed results in a null mutation, total RNA from the liver of embryos was analyzed by Northern blot hybridization (Figure 3A). A strong signal at 2.2kb corresponding to the *afp* transcript was detected in wild type and heterozygous embryos. No signal was detected in RNA samples extracted from homozygous embryonic liver, showing that the recombination disrupted the *afp* transcript in these animals. A Western blot was also performed on embryonic liver and amniotic fluid protein extracts (Figure 3B) and a strong signal was detected with the wild type extracts while no band corresponding to AFP was visible in the homozygous extracts demonstrating that these animals do not express AFP.

Table 1: Intercrosses

Strains	Parents		Offsprings		
	Male	Female	+/+	+/-	-/-
CD1	+/-	+/-	108	258	102
129	+/-	+/-	34	48	13
C57/black-6	+/-	+/-	19	43	19

AFP is required for female fertility

[0027] The Mendelian ratio obtained in CD1 and C57/black-6 background demonstrates that there is no reduction in the intercrosses. On the other hand, the divergence observed in 129 background suggests that AFP is involved in the gestation and that its importance is only revealed in some genetic contexts. In these litters derived from intercrossing heterozygous animals, homozygous embryos develop in the presence of their wild type and heterozygote littermates. AFP produced by these embryos is secreted and present in the maternal serum. To determine whether *afp* lacZ1/lacZ1 mice are able to develop in the complete absence of AFP, *afp* lacZ1/lacZ1 males and *afp* lacZ1/lacZ1 females were mated. No pups were obtained from these intercrosses suggesting an essential role of AFP for development and/or fertility (see Table 2). To test if fertility was affected, *afp* lacZ1/lacZ1 males and females were mated with wild type animals. Males homozygous for an *afp* disrupted allele appeared fertile and sired offspring but homozygous females never produce any live offspring. To test if natural matings occur with the *afp* lacZ1/lacZ1 females leave with wild type males the vaginal plug were checked. No plugs were detected with those females, showing that the origin of the observed infertility is due to an absence of mating. To identify the defect underlying the reproductive capacity of homozygous females, the

reproductive system of those animals was analyzed. The reproductive system of the *afp* lacZ1/lacZ1 females was dissected and at this stage of the analysis appeared complete. A major anatomical difference is notable between ovaries from *afp* lacZ1/lacZ1 and *afp* +/+ : the ovaries of *afp* lacZ1/lacZ1 are smooth, this observation suggests that those females do not ovulate. Histological analysis of mature *afp* lacZ1/lacZ1 ovaries shown that their homozygous tissues do not contain corpus lutea, the lack of these structure is indicative of the absence of ovulation (see Figure 4). The *afp* lacZ1/lacZ1 ovaries contain follicles at the different stages of maturation, this suggests that the default of AFP during the development has no effect on the female gametogenesis. However, the presence of follicles does not prove that these gametes are competent for maturation. To test the competence of the *afp* lacZ1/lacZ1 follicles, they were dissected out and analyzed their potential of maturation *in vitro*. Complete maturation was obtained *in vitro* with the dissected oocytes from *afp* lacZ1/lacZ1 animals. Taken together these data indicates that those females do not ovulate properly and thus that a signal needed to trigger ovulation is absent in the *afp* lacZ1/lacZ1 mice.

Table 2: Phenotypical analysis

Parents		Offsprings		
Male	Female	+/+	+/-	-/-
-/-	-/- (2)	0	0	0
-/-	+/+ (6)	0	71	0
+/+	-/- (13)	0	0	0

Table 2: Infertility phenotype of the *afp*lacZ1/lacZ1 (-/-) homozygous animals were mated, no offsprings were obtained

from these matings. To test fertility of the *afp*$^{lacZ1/lacZ1}$ (-/-) males and females, homozygous males and females were mated with wild type (+/+) animals. For the different breedings the number of mating is given in brackets.

[0028] It was also observed that *afp*$^{lacZ1/lacZ1}$ follicles are able to mature normally *in vitro* (data not shown) suggesting that the defect could be in a signal required to trigger ovulation and indeed ovulation can be induced in these animals by a superovulation protocol (Table 3).

Table 3: Ovulation induction in *afp*$^{lacZ1/+}$ and *afp*$^{lacZ1/lacZ1}$ females

Mice injected	Number of oocytes obtained
afp$^{lacZ1/+}$ females (9 weeks)	37
afp$^{lacZ1/lacZ1}$ females (9 weeks)	31

Table 3: Induction of ovulation in *afp*$^{lacZ1/lacZ1}$ females. *afp*$^{lacZ1/lacZ1}$ and *afp*$^{lacZ1/+}$ females were hormonally treated to induce ovulation. The average number of postovulation oocytes obtained from 6 individual females tested.

Mouse genotyping by PCR

[0029] In order to identify a possible mutation or deletion in the afp gene, a specific genotyping of afp -/- mice by PCR has been developed. Two primers are used for the first afp#1 anneals in the *afp* promoter region (-116 bp to -137 bp): according to the +1 of the mouse *afp* gene). The second primer afp#2 is complementary to the first exon of the mouse *afp* gene (+141 bp to +160 bp: according to the +1 of the mouse *afp* gene): sequence deleted in the *afp* -/- knock-out mouse described in the text.

Sequence afp#1: cccctgctctgttaattattg

Sequence afp#2: gaaaatagctcccaagtcac

[0030] No amplification product (300 bp) was observed in *afp* -/- animals. This amplification is present in wild type and heterozygous mice.

[0031] To differentiate +/- from wild type, a second PCR is performed using two primers giving an amplification on the DNA introduced in the genome of the transgenic animals (this sequence is not present in wild type animals).

[0032] For the knock-out mouse for *afp* described in the text: a couple of primers complementary to *lacZ* (*E. coli* gene) can be used.

lacZ#1: acaacgtcgtgactgggaaaac

lacZ#2: taatgggataggttacgt

[0033] Those primers will only give a signal (287 bp) on +/- and no signal on wild type DNA samples.

[0034] The physiological role of the alpha-fetoprotein, the most abundant serum protein expressed by mammalian embryos remains to be establish . This protein related to albumin excreted by the embryos into the maternal blood circulation has attracted attention and due to its abundance it has been postulated that the presence of this protein was essential for embryonic development. To determine the function of AFP this gene was disrupted by homologous recombination in embryonic stem cells. Surprisingly homozygous *afp*^{lacZ1}/lacZ1 are viable showing that expression of AFP by the embryo itself is not require for normal and complete development. This phenotype shows that the structure of the homozygous females was generally maintained. The different stages of ovocytes maturation are founded in these ovaries and shown to accomplish their maturation *in vitro*. Ovulation was induced in these females

by hormonal induction and ovocytes were produced. Although AFP is synthesized at a high level during fetal life (mainly by the liver and the visceral endoderm of the yolk sac), low level of AFP mRNA has been reported in different other fetal and adult tissues as well as in adult rats. However, the level of expression in such tissues is very low. Another explanation is that the ovulation in those females is affected by the lack of AFP during development.

[0035] The fact that in adults $afp^{lacZ1/lacZ1}$ ovulation can be induced argues for the absence in those females of a signal needed to trigger ovulation. This suggests that AFP is involved in the transportation of an element needed for ovulation in the adults.

[0036] By the disruption of the mouse AFP, one may show that fetal serum protein is required for females ovulation.

[0037] The fact that analbuminic rats are fertile shows that at least albumin cannot rescue AFP. This show that albumin and AFP plays two different role and that AFP is involved in the function of females ovaries.

[0038] The specific phenotype of the non-human mammals according to the invention is also illustrated in the enclosed figure 4, which presents the anatomical and histological analysis for the $afp^{lacZ1/lacZ1}$ ovaries:

A: Structure of the ovary (arrow) and uterus of an adult *afp*$^{lacZ1/lacZ1}$ (-/-) female;

B: Ovary from 12 weeks old *afp*$^{lacZ1/lacZ1}$;

C: Ovary from a 12 weeks old wild type female: the surface distortions are due to the presence of large type follicles, whereas *afp*$^{lacZ1/lacZ1}$ ovaries are smooth;

D: The general histological structure of the *afp*$^{lacZ1/lacZ1}$ ovaries is not affected and mature

Graafian follicles are present in those tissues (section from a fourth month old female);

E: At 4 months, the wild type ovaries exhibit large corpus lutea, indicative of successful ovulation. Those structures are never found in *afp*$^{lacZ1/lacZ1}$ ovaries.

[0039] Therefore, the non-human female mammals that present homozygously said mutation, partial or total deletion in the afp gene do not cycle. The surface of the ovaries is smooth and is not characterized by the presence of large follicles. Their histological structure is not generally affected, and Graafian follicles are identified in the tissues but no large corpus lutea is present.

[0040] Furthermore, females comprising an homozygous mutation or partial or total deletion in the *afp* gene do not allow uterus nidification of an embryo.

[0041] Additional experiments have shown that the *afp* gene is not essential for the survival of the mice. Indeed, as females give birth to severa animals, it was possible that the *afp*$^{-/-}$ may survive to brothers and sisters (*afp*$^{+/+}$ or *afp*$^{+/-}$) simultaneously present in the uterus of the female.

[0042] Therefore, in order to extrapolate the observed phenotype and genotype to the human population, the inventors have shown that blastocysts implanted one by one in pseudogravite females will obtain the birth of alive *afp*$^{-/-}$ animals that present the same phenotype as above-described (fertile males, sterile females).

[0043] Therefore, the *afp*$^{-/-}$ phenotype corresponds to alive sterile females, which is a phenotype that may exist in the mouse population as well as in the human population.

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CLAIMS

1. A non-human genetically modified mammal comprising a mutation, a partial or total deletion in the genetic sequence encoding the wild type mammal alpha-fetoprotein (AFP).

2. The non-human mammal according to claim 1, characterized in that it is a mouse.

3. The non-human mammal according to claim 1 or 2, characterized in that it comprises a heterozygous mutation, partial or total deletion in the genetic sequence encoding the wild type mammal alpha-fetoprotein (AFP).

4. The non-human mammal according to claim 1 or 2, characterized in that it comprises an homozygous mutation, partial or total deletion in the genetic sequence encoding the a wild type alpha-fetoprotein (AFP).

5. The non-human mammal according to claim 4, characterized in that it is a sterile female.

6. The non-human mammal according to claim 5; characterized in that it does not present a menstrual cyclization.

7. The non-human mammal according to claim 5, characterized in that it does not allow an uteral nidification of an embryo.

8. Use of the non-human mammal according to any one of the preceding claims, for the study, the testing and/or the screening of anti-osteoporosis fertilization and/or contraceptive methods, compounds and compositions.

9. A pluripotential embryonic stem cell, preferably a mouse cell, comprising a partial or total deletion of a genetic sequence encoding a mammal alpha-fetoprotein (AFP).

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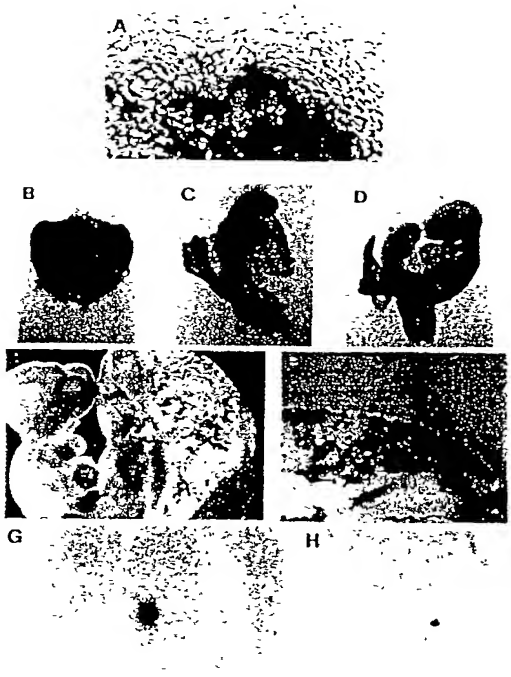
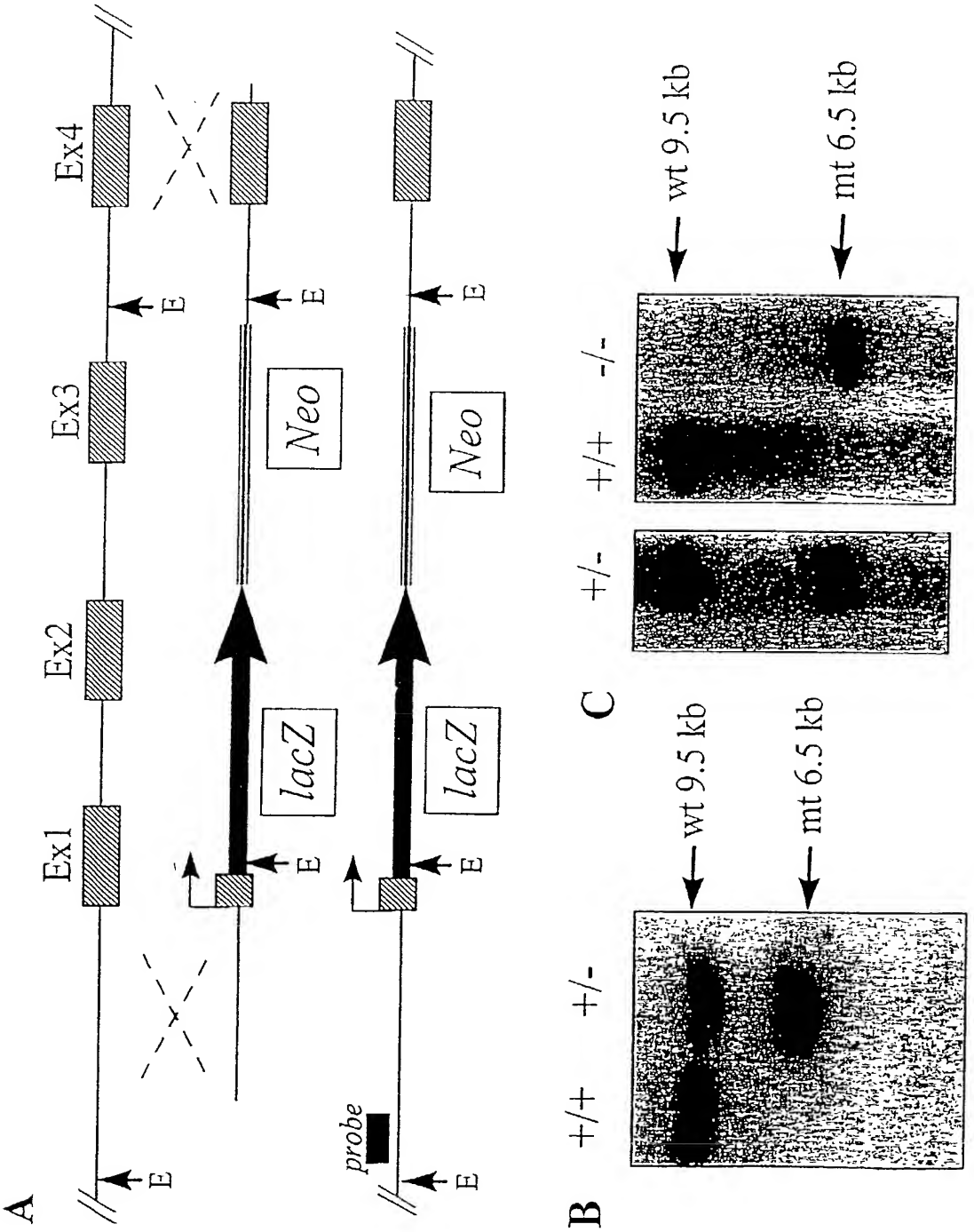
(54) Title: NON-HUMAN GENETICALLY MODIFIED MAMMAL LACKING THE ALPHA-FETOPROTEIN



(57) Abstract: The present invention is related to a non-human genetically modified mammal comprising a mutation, a partial or total deletion of the genetic sequence encoding the wild type mammal alpha-fetoprotein (AFP)

WO 01/03501 A2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lacZ
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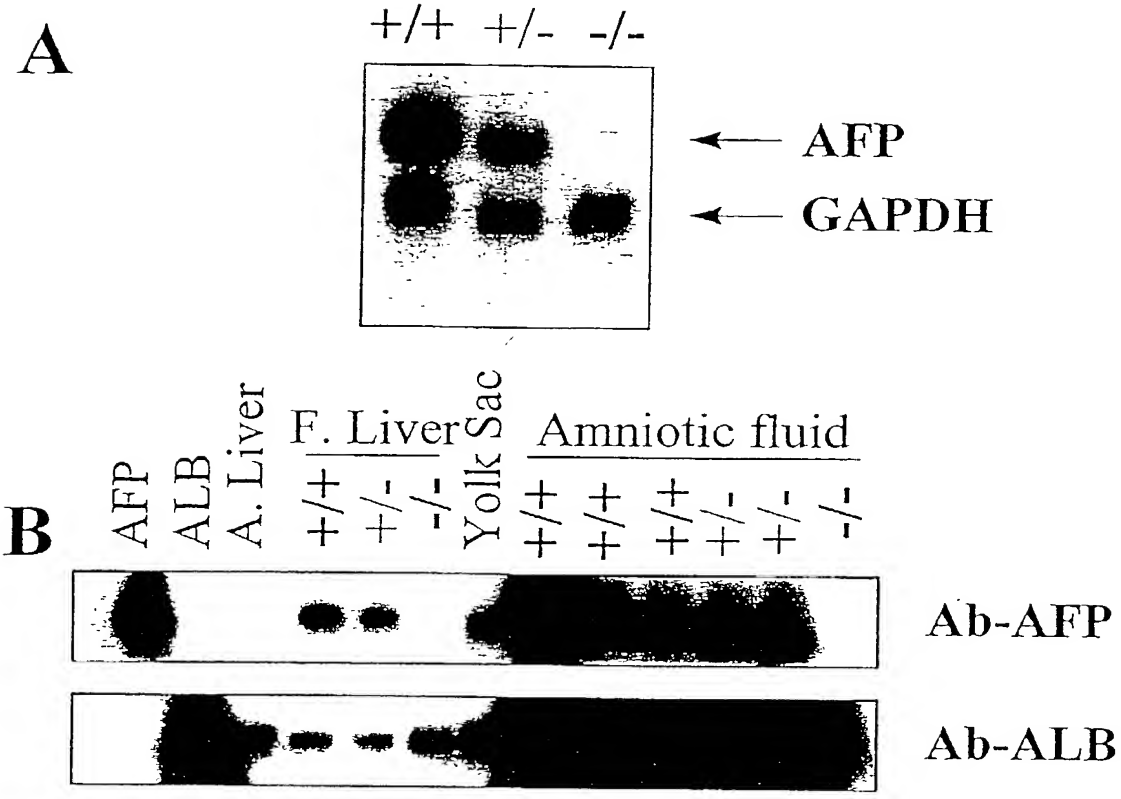
FIG.1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F. Liver
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Ab-AFP
Ab-ALB

FIG.2

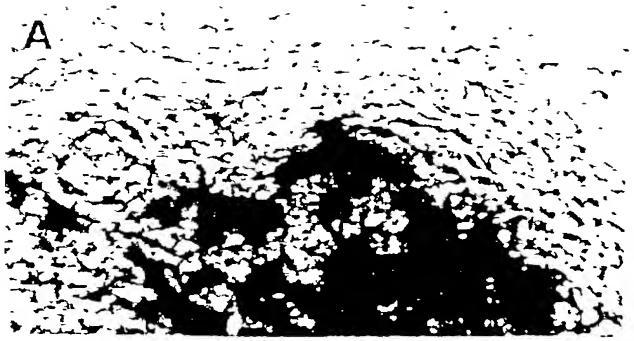
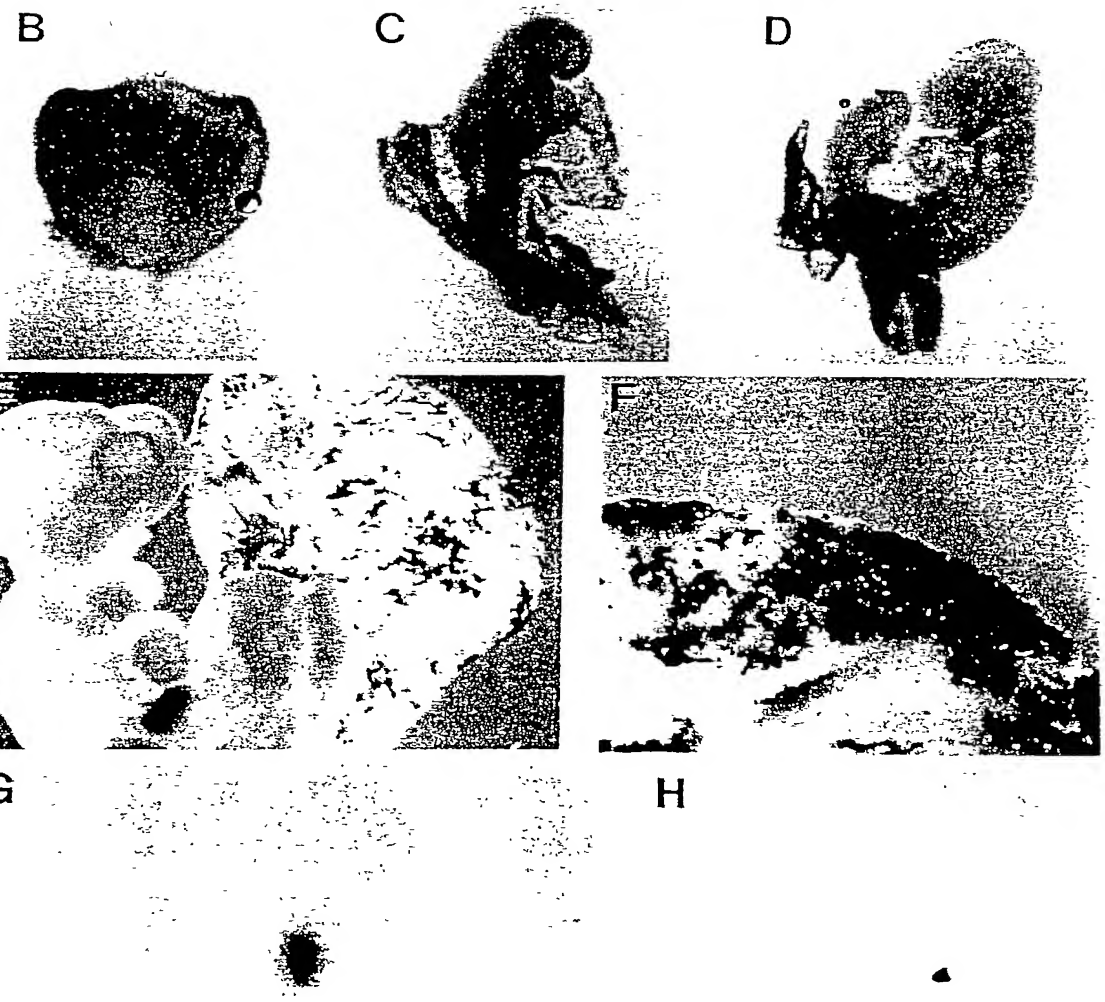


FIG. 3



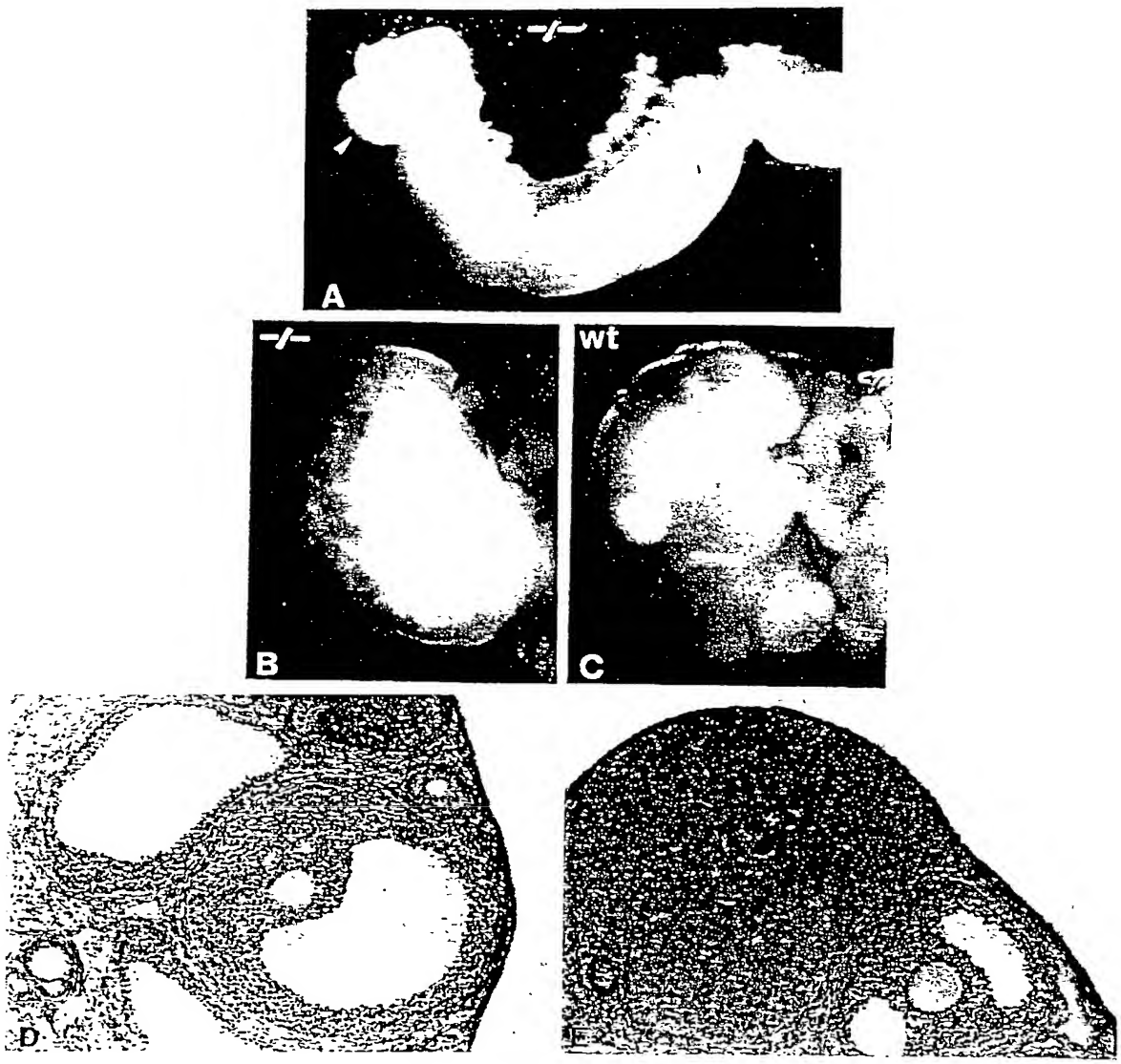


FIG. 4

Afp Genetic Sequence

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 121 ggatagcttc cacgttagat tcctcccagt gcgtgacgga gaagaatgtg cttagcatag
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Serial No.: 10/031,021
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PATENT
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AND
REVOCATION AND POWER OF ATTORNEY

To the Commissioner of Patents and Trademarks:

The undersigned is empowered to act on behalf of the assignee indicated below (the "Assignee"). The original assignment of the attached application for Letters Patent for the invention entitled **NON-HUMAN GENETICALLY MODIFIED MAMMAL LACKING THE ALPHA-FETOPROTEIN** (U.S. Patent Application Serial No. 10/031,021), from the inventors to the Assignee was submitted March 19, 2002, for recordation by the Assignment Branch. A **true copy** of this Assignment is attached hereto. This Assignment represents the entire chain of title of this invention from the Inventor(s) to the Assignee. I have reviewed this Assignment, and to the best of the Assignee's knowledge and belief, the Assignee is the owner of the entire right, title, and interest in the above-referenced application.

I declare that all statements made herein of my own knowledge are true, and that all statements made upon information and belief are believed to be true, and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that willful, false statements may jeopardize the validity of the application, or any patent issuing thereon.

The undersigned hereby revokes any previous powers of attorney in the subject application, and hereby appoints the registrants of Knobbe, Martens, Olson & Bear, LLP, 620 Newport Center Drive, Sixteenth Floor, Newport Beach, California 92660, Telephone (949) 760-0404, Customer No. 20,995, as its attorneys with full power of substitution and revocation to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected herewith. This appointment is to be to the exclusion of the inventor(s) and his attorney(s) in accordance with the provisions of 37 C.F.R. § 3.71.

Please use Customer No. 20,995 for all communications.

Assignee: UNIVERSITE LIBRE DE BRUXELLES

By: _____

Printed Name: _____

Title: Le Recteur
Pierre de MARET

Address: Avenue F.D. Roosevelt 50, CP 161
B-1050 Brussels BELGIUM

Dated: 15 -05- 2002

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Declaration and Power of Attorney for Patent Application

Déclaration et Pouvoirs pour demandes de brevet

French Language Declaration

En tant que l'inventeur nommé ci-après, je déclare par le présent acte que :

As a below named inventor, I hereby declare that :

Mon domicile, mon adresse postale et ma nationalité figurant ci-dessous à côté de mon nom,

My residence, post office address and citizenship are as stated below next to my name,

Je crois être le premier inventeur original et unique (si un seul nom est mentionné ci-dessous), ou l'un des premiers co-inventeurs originaux (si plusieurs noms sont mentionnés ci-dessous) du sujet revendiqué, pour lequel une demande de brevet a été déposée concernant l'invention intitulée :

I believe I am the original, first and sole (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled :

Non-human genetically modified mammal lacking the alpha-fetoprotein

et dont les caractéristiques sont fournies ci-joint à moins que la case suivante n'ait été cochée :

the specification of which is attached hereto unless the following box is checked :

O a été déposé le
sous le numéro de Demande des Etats-Unis ou
sous le numéro de demande internationale PCT
et modifiée le
(le cas échéant).

X was filed on January 14, 2002
as United States Application Number or PCT International Application Number
10/031,021
and was amended on
(if applicable).

Je déclare par le présent acte avoir passé en revue et pris connaissance du contenu des caractéristiques ci-dessus, revendications comprises, telles que modifiées par tout amendement dont il aura été fait référence ci-dessus.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

Je reconnais de voir divulguer toute information pertinente à l'examen de cette demande, comme le définit le Titre 37, §1.56 du Code fédéral des réglementations.

I acknowledge the duty to disclose information which is material to patentibility as defined in Title 37, Code of Federal Regulations, § 1.56.

French Language Declaration

Je revendique par le présent acte avoir la priorité étrangère, en vertu du Titre 35, § 119 du Code des Etats-Unis, sur toute demande étrangère de brevet ou certificat d'inventeur figurant ci-dessous et ai aussi pris connaissance de toute demande étrangère de brevet ou de tout certificat d'inventeur ayant une date de dépôt précédant celle de la demande à propos de laquelle une priorité est revendiquée.

I hereby claim foreign priority under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior foreign applications Demande(s) de brevet antérieure(s)		Priority claimed Droit de priorité revendiqué	
60/143,269	**United States**	●	○
(Number) (Numéro)	(Country) (Pays)	Yes Oui	No Non
		○	○
(Number) (Numéro)	(Country) (Pays)	Yes Oui	No Non
		○	○
(Number) (Numéro)	(Country) (Pays)	Yes Oui	No Non

12 July 1999
(Day/Month/Year Filed)
(Jour/Mois/Année de dépôt)

Je revendique par le présent acte tout bénéfice, en vertu du Titre 35, § 120 du Code des Etats-Unis, de toute demande de brevet effectuée aux Etats-Unis figurant ci-dessous et, dans la mesure où le sujet de chacune des revendications de cette demande de brevet n'est pas divulgué dans la demande américaine préalable, en vertu des dispositions de premier paragraphe du Titre 35, § 112 du Code des Etats-Unis, je reconnais devoir divulguer toute information pertinente à la demande de brevet comme défini dans le Titre 37, § 1.56 du Code fédéral des réglementations, dont j'ai pu disposer entre la date de dépôt de la première demande et la date de dépôt de la demande nationale ou PCT internationale :

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentibility as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application :

(Application Serial No.) (No. de série de la demande)	(Filing date) (Date de dépôt)	(Statut) (Breveté, en attente, annulé)	(Status) (Patented, pending, abandoned)
(Application Serial No.) (No. de série de la demande)	(Filing date) (Date de dépôt)	(Statut) (Breveté, en attente, annulé)	(Status) (Patented, pending, abandoned)

Je déclare par le présent acte que toute déclaration ci-incluse est, à ma connaissance, véridique et que toute déclaration formulée à partir de renseignements ou de suppositions est tenue pour véridique; et de plus, que toutes ces déclarations ont été formulées en sachant que toute fausse déclaration volontaire ou son équivalent est passible d'une amende ou d'une incarcération, ou des deux, en vertu de la Section 1001 du Titre 18 du Code des Etats-Unis et que de telles déclarations volontairement fausses risquent de compromettre la validité de la demande de brevet ou du brevet délivré à partir de celle-ci.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful and false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

French Language Declaration

POUVOIRS : En tant que l'inventeur cité, je désigne par la présente l'(les) avocat(s) et/ou agent(s) suivant(s) pour qu'il(s) poursuive(nt) la procédure de cette demande de brevet et traite(nt) toute affaire avec le Bureau des brevets et marques s'y rapportant.
(mentionner le nom et le numéro d'enregistrement)

POWER OF ATTORNEY : As named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and trademark Office connected there with.

(list name and registration number)

Daniel Hart, Reg. No.: 40,637

Adresser toute correspondance à :

Send Correspondence to :

Knobbe, Martens, Olson & Bear, LLP
620 Newport Center Dr., 16th Flr.
Newport Beach, CA 92660

Adresser tout appel téléphonique à :
(nom et numéro de téléphone)

Direct Telephone Calls to :
(name and telephone number)

Daniel Hart, (619) 235-8550

Nom complet de l'unique ou premier inventeur 1-00	Full name of sole or first inventor ***GABANT Philippe***
Signature de l'inventeur Date	Inventor's signature Date - 5 FEB. 2002
Domicile	Residence ***Rue Van Bemmel, 9*** ***B-1210 BRUSSELS*** ***BELGIUM*** BEX
Nationalité	Citizenship ***Belgian***
Adresse postale	Post Office Address ***Rue Van Bemmel, 9*** ***B-1210 BRUSSELS*** ***BELGIUM***

Nom complet du second co-inventeur, le cas échéant 2-00	Full name of second joint inventor, if any ***ROSCAM-SZPIRER Josiane***
Signature du second inventeur Date	Second inventor's signature Date - 5 FEB. 2002
Domicile	Residence ***Avenue Jassogne, 27*** ***B-1410 WATERLOO*** ***BELGIUM*** BEX
Nationalité	Citizenship *Belgian*
Adresse postale	Post Office Address ***Avenue Jassogne, 27*** ***B-1410 WATERLOO*** ***BELGIUM***

(Fournir les mêmes renseignements et la signature de tout co-inventeur supplémentaire)

(Supply similar information and signature for any subsequent joint inventor)